Amendments to the Specification

Please replace paragraph [0001] with the following amended paragraph:

[0001] The present application is a continuation of U.S. Application No. 10/454,793, filed June 5, 2003, which claims the benefit of the filing date of United States provisional patent application number 60/385,613, filed June 5, 2002. ~~This application~~ U.S. Application No. 10/454,793 also is a continuation-in-part of U.S. Application No. 10/005,876, filed December 7, 2001, which claims the benefit of the filing dates of U.S. provisional application numbers 60/333,124, filed November 27, 2001, and 60/318,902, filed September 14, 2001,[[.]] ~~This application~~ and also is a continuation-in-part of U.S. Application No. 10/014,128, filed December 7, 2001, and of U.S. Application No. 09/732,914, filed December 11, 2000. The disclosures of all of the above-referenced applications are specifically incorporated herein by reference in their entireties.

Please replace paragraph [0111] with the following amended paragraph:

[0111] Figures 8A and 8B depict generating a covalently linked double stranded nucleotide sequence containing an element on each end according to a method of the invention. "PCR" indicates polymerase chain reaction; "TOPO" indicates topoisomerase; topoisomerase shown as circle attached to sequence; "P1" and "P2" indicate PCR primers. Topoisomerase recognition site is indicated in bold print. (5'-CGGAACAAGGG (SEQ ID NO: 63); 3'-GGGAACCGGAT (SEQ ID NO: 64); 5'-CCCTTCGGAACAAGGG (SEQ ID NO: 65); 5'-CCCTTGGCCATAAGGG (SEQ ID NO: 66); 5'-GGCCATAAGGG (SEQ ID NO: 135); 3'-GGGAAGCCTTG (SEQ ID NO: 136))

Please replace paragraph [0125] with the following amended paragraph:

[0125] Figure 18 is a map of the multiple cloning site in plasmids pcDNAGW-DT(sc) and pENTR-DT(sc). (SEQ ID NO: 67; amino acid sequences SEQ ID NO: 68 and SEQ ID NO: 69)

Please replace paragraph [0129] with the following amended paragraph (Note that underlined web address has not been added. It was underlined in the original text.):

[0129] Figure 22 is a depiction of the physical map (Fig. 22A) showing the TOPO cloning site in, and the nucleotide sequence (Fig. 22B (SEQ ID NO: 70)) of, plasmid pENTR/D-TOPO. The physical map depicts the adapted, supercoiled form of the vector, while the nucleotide sequence depicts the vector containing a start codon and an open reading frame (atgnnnnnn...). Restriction sites are labeled to indicate the actual cleavage site. The boxed region indicates *att*L sequences in the entry clone that will be transferred into the destination vector following recombination. The sequence of pENTR/D-TOPO depicted in Figure 22B is also available for downloading from the Invitrogen Corporation web site at http://www.invitrogen.com./content/vectors/pentr_dtopo_seq.txt.

Please replace paragraph [0130] with the following amended paragraph:

[0130] Figure 23 is a depiction of the physical map (Fig. 23A) showing the TOPO cloning site in, and the nucleotide sequence (Fig. 23B (SEQ ID NO: 71)) of, plasmid pENTR/SD/D-TOPO. The physical map depicts the adapted, supercoiled form of the vector, while the nucleotide sequence depicts the vector containing a start codon and an open reading frame (atgnnnnnn...). Restriction sites are labeled to indicate the actual cleavage site. The boxed region indicates *att*L sequences in the entry clone that will be transferred into the destination vector following recombination. The nucleotide sequence of pENTR/SD/D-TOPO depicted in Figure 23B is also available for downloading from http://www.invitrogen.com./content/vectors/pentrsd_dtopo_seq.txt.

Please replace paragraph [0131] with the following amended paragraph:

[0131] Figure 24 is a depiction of the physical map (Fig. 24A) and the nucleotide sequence (Fig. 24B-C) (SEQ ID NO: 72) of plasmid pcDNA3.2/V5/GWD-TOPO7. The physical map depicts the adapted, supercoiled form of the vector, while the nucleotide

sequence depicts the vector containing a start codon and an open reading frame (atgnnnnnn...).

Please replace paragraph [0132] with the following amended paragraph:

[0132] Figure 25 is a depiction of the physical map (Fig. 25A) and the nucleotide sequence (Fig. 25B-C) (SEQ ID NO: 73) of plasmid pcDNA6.2/V5/GWD-TOPO7. The physical map depicts the adapted, supercoiled form of the vector, while the nucleotide sequence depicts the vector containing a start codon and an open reading frame (atgnnnnnn...).

Please replace paragraph [0133] with the following amended paragraph:

[0133] Figure 26 is a depiction of an exemplary adaptation strategy for pENTR/SD-dTopo (SEQ ID NO: 74: SEQ ID NO: 75; SEQ ID NO: 138; SEQ ID NO 139), pENTR-dTopo (SEQ ID NO: 76; SEQ ID NO: 78; SEQ ID NO: 140; SEQ ID NO: 141), and pcDNAGW-dTopo (SEQ ID NO: 77; SEQ ID NO: 79; SEQ ID NO: 142).

Please replace paragraph [0136] with the following amended paragraph:

[0136] Figure 29 is a schematic depiction of the binding of a topoisomerase to a recognition site near the 3' terminus of a target nucleic acid molecule. Upon binding of the topoisomerase, the downstream sequence (3' to the cleavage site) can dissociate, leaving a nucleic acid molecule having the topoisomerase covalently bound to the newly generated 3' end. (SEQ ID NO: 80)

Please replace paragraph [0198] with the following amended paragraph:

[0198] Sites that may be used in the present invention include att sites. The 15 bp core region of the wildtype att site (GCTTTTTTAT ACTAA (SEQ ID NO: 81)), which is identical in all wildtype att sites, may be mutated in one or more positions. The inventors have determined that *att* sites that specifically recombine with other *att* sites can be constructed by altering nucleotides in and near the 7 base pair overlap region,

bases 6-12 of the core region. Thus, recombination sites suitable for use in the methods, compositions, and vectors of the invention include, but are not limited to, those with insertions, deletions or substitutions of one, two, three, four, or more nucleotide bases within the 15 base pair core region (*see* U.S. Application Nos. 08/663,002, filed June 7, 1996 (now U.S. Patent No. 5,888,732) and 09/177,387, filed October 23, 1998, which describes the core region in further detail, and the disclosures of which are incorporated herein by reference in their entireties). Recombination sites suitable for use in the methods, compositions, and vectors of the invention also include those with insertions, deletions or substitutions of one, two, three, four, or more nucleotide bases within the 15 base pair core region that are at least 50% identical, at least 55% identical, at least 60% identical, at least 65% identical, at least 70% identical, at least 75% identical, at least 80% identical, at least 85% identical, at least 90% identical, or at least 95% identical to this 15 base pair core region.

Please replace paragraph [0201] with the following amended paragraph:

[0201] The core sequence of each *att* site (*att*B, *att*P, *att*L and *att*R) can be divided into functional units consisting of integrase binding sites, integrase cleavage sites and sequences that determine specificity. Specificity determinants are defined by the first three positions following the integrase top strand cleavage site. These three positions are shown with underlining in the following reference sequence: CAACTTTTTTATAC AAAGTTG (SEQ ID NO: 82). Modification of these three positions (64 possible combinations) can be used to generate *att* sites that recombine with high specificity with other *att* sites having the same sequence for the first three nucleotides of the seven base pair overlap region. The possible combinations of first three nucleotides of the overlap region are shown in Table 1.

Please replace current pages 89-91 with amended pages 89-91 that are appended hereto.

Please replace paragraph [0359] with the following amended paragraph (Note that underlined portions of sequences have not been added. They were underlined in the original text.):

[0359] Shuman teaches that Vaccinia topoisomerase binds to duplex DNA and cleaves the phosphodiester backbone of one strand while exhibiting a high level of sequence specificity. Cleavage occurs at a consensus pentapyrimidine element 5'-(C/T)CCTT-3' or related sequences in the scissile strand. In one embodiment the scissile bond is situated in the range of 2-12 bp from the 3' end of the duplex DNA. In another embodiment cleavable complex formation by Vaccinia topoisomerase requires six duplex nucleotides upstream and two nucleotides downstream of the cleavage site. Examples of Vaccinia topoisomerase cleavable sequences include, but are not limited to, +6/-6 duplex GCCCTTATTCCC (SEQ ID NO: 29), +8/-4 duplex TCGCCCTTATTC (SEQ ID NO: 30), +10/-2 duplex TGTCGCCCTTAT (SEQ ID NO: 31), +11/-1 duplex GTGTCGCCCTTA (SEQ ID NO: 32).

Please replace paragraph [0428] with the following amended paragraph:

[0428] The invention also relates to host cells, or derivatives thereof, comprising one or more of the nucleic acid molecules or vectors of the invention, particularly those nucleic acid molecules and vectors described in detail herein. Representative host cells that may be used according to this aspect of the invention include, but are not limited to, bacterial cells, yeast cells, plant cells and animal cells, and derivatives thereof. Preferred bacterial host cells include Escherichia spp. cells (particularly E. coli cells and most particularly E. coli strains DH10B, Stbl2, DH5α, DB3, DB3.1 (preferably E. coli LIBRARY EFFICIENCY® DB3.1™ Competent Cells; Invitrogen Corporation, Carlsbad, CA), DB4, DB5, JDP682 and ccdA-over (see U.S. Application No. 09/518,188, filed March 2, 2000, and U.S. provisional Application No. 60/475,004, filed June 3, 2003, by Louis Leong *et al.*, entitled "Cells Resistant to Toxic Genes and Uses Thereof," the disclosures of which are incorporated by reference herein in their entireties); Bacillus spp. cells (particularly

B. subtilis and B. megaterium cells); Streptomyces spp. cells; Erwinia spp. cells; Klebsiella spp. cells; Serratia spp. cells (particularly S. marcessans cells); Pseudomonas spp. cells (particularly P. aeruginosa cells); and Salmonella spp. cells (particularly S. typhimurium and S. typhi cells). Preferred animal host cells include insect cells (most particularly Drosophila melanogaster cells, Spodoptera frugiperda Sf9 and Sf21 cells and Trichoplusa High-Five cells), nematode cells (particularly C. elegans cells), avian cells, amphibian cells (particularly Xenopus laevis cells), reptilian cells, and mammalian cells (most particularly NIH3T3, CHO, COS, VERO, BHK and human cells). Preferred yeast host cells include Saccharomyces cerevisiae cells and Pichia pastoris cells. In addition, derivatives of such host cells are suitable for use in accordance with the present invention. These and other suitable host cells are available commercially, for example from Invitrogen Corporation (Carlsbad, California), American Type Culture Collection (Manassas, Virginia), and Agricultural Research Culture Collection (NRRL; Peoria, Illinois).

Please replace paragraph [0503] with the following amended paragraph (Note that underlined portions of sequences have not been added. They were underlined in the original text.):

[0503] The first adapter oligonucleotide, (TOPO D1), has complementation to the EcoRI cohesive end, 3'-TTAA-5'. Furthermore, TOPO D1 has an additional 24-bp including the topoisomerase consensus pentapyrimidine element 5'-CCCTT located 16-bp upstream of the 3' end. The remaining sequence and size of TOPO D1 adapter oligo is variable, and may be modified to fit a researcher's particular needs. According to one such aspect of this preferred embodiment of the invention, 5'-AATTGATCCCTTCACCGACATAGTACAG-3 (SEQ ID NO: 33) is the full sequence of the adapter used.

Please replace paragraph [0504] with the following amended paragraph (Note that underlined portions of sequences have not been added. They were underlined in the original text.):

[0504] The second adapter oligonucleotide, (TOPO D2), must have full complementation to TOPO D1. TOPO D2 complements directly 5′ of the EcoRI cohesive flap, extending the bottom strand of the linearized vector. Additionally, TOPO D2 contains the sequence 3′-GTGG, which is the necessary SSS for directional cloning. In this embodiment, the SSS was chosen to complement the Kozak sequence known to help expression of ORFs in eukaryotic cells by increasing the efficiency of ribosome binding on the mRNA, however, sequence and length are highly variable to meet the specific needs of individual users. The complete sequence of TOPO D2 is 3-CTAGGGAAGTGG-5 (SEQ ID NO: 34) . Similar to above, the adapter duplex that results from the annealing of oligonucleotides TOPO D4 and TOPO D5 has a single-stranded SacI overhang at one end, and a 12 nucleotide single-stranded overhang at the other end.

Please replace paragraph [0505] with the following amended paragraph (Note that underlined portions of sequences have not been added. They were underlined in the original text.):

[0505] The third adapter oligonucleotide (TOPO D5), has complementation to the SacI cohesive end, 3'-TCGA-5'. Similar to TOPO D1, TOPO D5 has additional bases creating a single stranded overhang. The length and sequence can vary based on the needs of the user. In the current embodiment TOPO D5's sequence is 5'-AAGGGCGAGCT-3' (SEQ ID NO: 35).

Please replace paragraph [0506] with the following amended paragraph (Note that underlined portions of sequences have not been added. They were underlined in the original text.):

[0506] The fourth adapter oligonucleotide (TOPO D4), has full complementation to TOPO D5, and complements directly 5′ of the SacI cohesive flap extending the top strand of the linearized vector. TOPO D4 also contains the topoisomerase consensus sequence 5′-CCCTT. The remaining sequence and size of TOPO D4 adapter oligo is variable and may be modified to fit particular needs. In the current embodiment, the sequence of TOPO D4 is 3'-GACATGATACAGTTCCCGC-5' (SEQ ID NO: 36), which includes an additional 12 bp single stranded overhang.

Please replace paragraph [0510] with the following amended paragraph (Note that underlined portions of sequences have not been added. They were underlined in the original text.):

[0510] The annealing oligonucleotide (TOPO D3), must have complementation to the single stranded DNA overhangs of TOPO D1 and TOPO D4. In the current embodiment the overhangs both share the following sequence, 5′-GACATAGTACAG-3' (SEQ ID NO:37). Therefore, TOPO D3 has the following sequence, 3-CTGTATCATGTCAAC-5 (SEQ ID NO: 38), which comprises full complementation to the adapter oligos' single stranded overhang and an additional 3 bp overhang, 3′-AAC-5′.

Please replace paragraph [0519] with the following amended paragraph (Note that underlined portions of sequences have not been added. They were underlined in the original text.):

[0519] The first adapter oligonucleotide, (TOPO H), has complementation to the HindIII cohesive end, 3'-TCGA-5'. Furthermore, TOPO H has an additional 24 bp including the topoisomerase consensus pentapyrimidine element 5′-CCCTT located 19-bp upstream of

the 3' end. The remaining sequence and size of TOPO H adapter oligo is variable, and may be modified to fit a researcher=s particular needs. In the current embodiment 5'-AGCTCGCCCTTATTCCGATAGTG-3' (SEQ ID NO: 39) is the full sequence of the adapter used.

Please replace paragraph [0520] with the following amended paragraph (Note that underlined portions of sequences have not been added. They were underlined in the original text.):

[0520] The second adapter oligonucleotide (TOPO 16), must have full complementation to TOPO H. TOPO 16 complements directly 5' of the HindIII cohesive end, extending the bottom strand of the linearized vector. Additionally, TOPO 16 contains the sequence 3'-TAAG, which is the chosen single stranded sequence for directional cloning. The complete sequence of TOPO 16 is 3'-GCGGGAATAAG-5' (SEQ ID NO: 40).

Please replace paragraph [0521] with the following amended paragraph:

[0521] The third adapter oligonucleotide (TOPO 1), has complementation to the EcoRI cohesive end, 3'-TTAA-5'. Similar to TOPO H, TOPO 1 has additional bases containing the topoisomerase I consensus sequence CCCTT located 12 bp upstream of the 3' end. The length and sequence of TOPO 1 can vary based on the needs of the user. In the current embodiment TOPO 1's sequence is 5'-AATTCGCCCTTATTCCGATAGTG-3' (SEQ ID NO: 41).

Please replace paragraph [0522] with the following amended paragraph:

[0522] The fourth adapter oligonucleotide (TOPO 2), has full complementation to TOPO 1, and complements directly 5' of the EcoRI cohesive end extending the top strand of the linearized vector. In the current embodiment, the sequence of TOPO 2 is 3'-GCGGGAA-5' ~~(SEQ ID NO:)~~.

Please replace paragraph [0525] with the following amended paragraph:

[0525] The annealing oligonucleotide (TOPO 17), must have complementation to the single stranded DNA overhang of TOPO H. In the current embodiment the overhang has the following sequence, 5'-CGATAGTG-3' ~~(SEQ ID NO:)~~. Therefore, TOPO 17 has the following sequence, 3'-GCTATCAC-5' ~~(SEQ ID NO:)~~, which comprises full complementation to the adapter oligo's single stranded overhang.

Please replace paragraph [0526] with the following amended paragraph (Note that underlined portions of sequences have not been added. They were underlined in the original text.):

[0526] The annealing oligonucleotide (TOPO 3), must have complementation to the single stranded DNA overhang of TOPO 1. In the current embodiment the overhang has the following sequence, 3'-GTGATAGCCTTA-5' (SEQ ID NO:42). Therefore, TOPO 3 has the following sequence, 5'-CAACACTATCGGAAT-3' (SEQ ID NO: 43), which comprises full complementation to the adapter oligo's single stranded overhang and an additional 3 bp overhang, 5'-CAA-3'.

Please replace paragraph [0549] with the following amended paragraph (Note that underlined portions of sequences have not been added. They were underlined in the original text.):

[0549] Example of Forward Primer Design. Below is the DNA sequence of the N-terminus of a theoretical protein and the proposed sequence for a corresponding forward PCR primer. The ATG initiation codon is underlined.

DNA sequences:, 5'-ATG GGA TCT GAT AAA (SEQ ID NO: 122)

Proposed Forward PCR primer: 5'-CACC ATG GGA TCT GAT AAA (SEQ ID NO: 123)

Please replace paragraph [0555] with the following amended paragraph (Note that underlined portions of sequences have not been added. They were underlined in the original text.):

[0555] Example A of Reverse Primer Design. Below is the sequence of the C-terminus of a theoretical protein. The protein should be fused in frame with a C-terminal tag (following recombination of the entry clone with a GATEWAY™- destination vector). The stop codon is underlined.

DNA sequence: AAG TCG GAG CAC TCG ACG ACG GTG TAG-3' (SEQ ID NO: 46)

One solution is to design the reverse PCR primer to start with the codon just upstream of the stop codon, but the last two codons contain GTGG (underlined below), which is identical to the 4 bp overhang sequence. As a result, the reverse primer will be complementary to the 4 bp overhang sequence, increasing the probability that the PCR product will clone in the opposite orientation. This situation should be avoided.

DNA sequence: AAG TCG GAG CAC TCG ACG ACG GTG TAG-3' (SEQ ID NO: 46)

Proposed Reverse PCR primer sequence: TG AGC TGC TGC CAC AAA-5' (SEQ ID NO: 47)

Please replace paragraph [0557] with the following amended paragraph (Note that underlined portions of sequences have not been added. They were underlined in the original text.):

[0557] Example B of Reverse Primer Design. Below is the sequence for the C-terminus of a theoretical protein. The stop codon is underlined.

...GCG GTT AAG TCG GAG CAC TCG ACG ACT GCA TAG-3´(SEQ ID NO: 48)

To fuse the ORF in frame with a C-terminal tag (supplied by the destination vector after recombination), remove the stop codon by starting with nucleotides homologous to the last codon (TGC) and continue upstream. The reverse primer will be:

5'-TGC AGT CGT CGA GTG CTC CGA CTT-3' (SEQ ID NO: 49)

This will amplify the C-terminus without the stop codon and allow the ORF to be joined in frame with a C-terminal tag. If it is not desirable to join the ORF in frame with a C-terminal tag, the reverse primer should simply be designed to include the stop codon:

5'-CTA TGC AGT CGT CGA GTG CTC CGA CTT-3' (SEQ ID NO: 50)

Important: It must be remembered that the pENTR TOPO® vectors accept blunt-end PCR products. 5' phosphates should not be added to the primers for PCR, as this will prevent ligation into the pENTR TOPO® vectors. In addition, it is recommended that the oligonucleotides be gel-purified prior to use, especially if they are long (> 30 nucleotides).

Please replace current page 287 with amended page 287 that is appended hereto.

Please replace paragraph [0679] with the following amended paragraph:

[0679] The following oligos were synthesized and gel-purified:

T7topG
5'-pGACTCGTAATACGACTCACTATAGGGCCCTTATTCCGATAGTG-3' (SEQ ID NO: 124)

T7botG
pAGGGCCCTATAGTGAGTCGTATTACGAGTCAAAAAAAAAAAA (SEQ ID NO: 125)

TOPO-5 pCAACACTATCGGAATA (SEQ ID NO: 126)

Please replace paragraph [0686] with the following amended paragraph:

[0686] pBAD/TOPO-actin-as was created by TOPO cloning a blunt PCR product amplified with an actin forward, actinF, primer having the sequence 5'-GCTCACCATGGATGATGATATCGC-3' (SEQ ID NO: 127) and an actin reverse, actinR, primer having the sequence 5'-GGAGGAGCAATGATCTTGATCTTC-3' (SEQ ID NO: 128) from the HeLa cDNA PCR control template (available from Invitrogen Corporation, Carlsbad CA, catalog number 46-0324) into pBAD/TOPO in the antisense orientation.

Please replace paragraph [0687] with the following amended paragraph:

[0687] pUC19/actin (Figure 46A) was created by cloning of a BamHI-HindIII digested PCR product amplified from the HeLa cDNA template with BamHI-actinF primer having the sequence 5'-CACGGATCCGCTCACCATGGATGATGATATCGC-3' (SEQ ID NO: 129) and actinR-HindIII primer having the sequence 5'-CACAAGCTTGGAGGAGCAATGATCTTGATCTTC (SEQ ID NO: 130) into BamHI-HindIII digested pUC19.

Please replace paragraph [0688] with the following amended paragraph:

[0688] 50µl reactions were used for both primary and secondary amplifications using 10pmol each primer, 0.2mM dNTPs, 1X PCR buffer (from 10X stock, Invitrogen Corporation, Carlsbad CA), and 2.5U Platinum Taq DNA polymerase or Recombinant Taq DNA polymerase. Primary reactions were performed using 1ng of pBAD/TOPO-actin-as, pUC19/actin, or pcDNA5/FRT/TO/GFP plasmids as templates and actinF + actinR or GFPstart (5'-ATGGCTAGCAAAGGAGAAGAACTTT-3' (SEQ ID NO: 131)) + GFPstop2 (5'-TTATTTGTAGAGCTCATCCATGCCA-3' (SEQ ID NO: 132)) primers. For transcription control templates, the GFP and actin forward primers were paired with reverse primers appended with a 5' T7 promoter sequence (5'-GATGACTCGTAATACGACTCACTATAGGG-3' (SEQ ID NO: 133)). Secondary

reactions were the same as the primary reactions except 1μl of T7 TOPO linking reaction was used as template and either actinF or GFPstart primers were combined with the linker-specific primer T7amp1 (5'-GATGACTCGTAATACGACTCACTA-3' (SEQ ID NO: 134)).

In the specification, after the Abstract of the Disclosure at page 311 and before the drawings, please insert the Sequence Listing (pages 1-38) that is appended hereto.